Molecular and morpho-physiological characterization of sea, ruderal and cultivated beets

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Abstract *Beta vulgaris* genetic resources are essential for broadening genetic base of sugar beet and developing cultivars adapted to adverse environmental conditions. Wild beets (sea beets, *B. vulgaris* spp. *maritima* and their naturalized introgressions with cultivated beets known as ruderal beets) harbor substantial genetic diversity that could be useful for beet improvement. Here, we compared molecular and morpho-physiological traits of wild beets collected on the Adriatic coast of Italy with sugar beet using eight primer-pairs amplifying 194 polymorphic fragments and four root traits (glucose and fructose content in the root tip, root elongation rate, number of the of root tips, total root length and its distribution among diameters ranges). Genetic diversity was higher in the sea beet accession, which may be due to the highly variable selection pressures that occur in heterogeneous ecological niches, compared with the ruderal and cultivated beets. Sea and sugar beet accessions showed contrasting root patterns in response to sulfate deprivation: sugar beet showed an increase of reducing sugars in the root tips and higher root elongation rate, and the sea beet accession showed an increase in root tip number, total root length and fine root length (average diameter < 0.5 mm). The ruderal beet showed intermediary responses to sea and sugar beet accessions. AFLP and morpho-physiological cluster analyzes showed sea, ruderal and cultivated beets to be genetically distinct groups. The results of this study indicate variability in response to sulfate deprivation is present in undomesticated beets that could be deployed for sugar beet improvement.

Keywords Genetic resources · Root plasticity · Nutritional stress · Sulfate deprivation · AFLP

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Introduction

Yield of sugar beet (*Beta vulgaris* L. ssp. *vulgaris*, Sugar Beet Group) strongly depends on environmental conditions (Kenter et al. 2006) and marked decreases in sucrose production occur under limited water and nutrient availability (Freckleton et al. 1999; Shaw

et al. 2002). Global change models predict more severe crop yield losses due to these environmental stresses in Central and Southern Europe (Jones et al. 2003), and as a consequence, the development of more stress-tolerant sugar beet varieties is urgently needed. The narrow gene pool of sugar beet increases vulnerability to abiotic stresses, emphasizing the need to broaden its genetic base by introgression of novel stress-tolerance genes (Ober and Luterbacher 2002; Panella and Lewellen 2007). In addition to cultivated beets, *B. vulgaris* includes several interfertile forms which represent a largely unexploited source of genetic diversity (Desplanque et al. 1999) including sea beets [*B. vulgaris* ssp. *maritima* (L.) Arcang.] common along the European coasts, ruderal beets present in disturbed inland habitats, and weed beets that infest sugar-beet fields. In the past, the sea beet subspecies have been a useful source of genetic diversity for improving cultivated beets, particularly for disease resistance traits (Van Geyt et al. 1990; Biancardi et al. 2002). Exploiting wild and cultivated *Triticum* germplasm for root characteristics has identified stress avoidance characters under conditions of limited water and nutrient availability (Reynolds et al. 2007; Waines and Ehdaie 2007) and in *Poa pratensis* L., Sullivan et al. (2000) demonstrated substantial genetic variability for root traits including total length, total area, average diameter, and length distribution among root thickness classes correlated with differences in their adaptation to nutritional stress. Detection of genetic variation for root traits is an active area in breeding for nutritional stress tolerance (Miller et al. 2003). Studies in maize (*Zea mays* L.) and sugar beet dissecting root system into morpho-functional components demonstrated good correlations between the uptake of sulfate following deprivation, root elongation rate, root length density, root proliferation, and crop yield (Saccomani et al. 1981; Vamerali et al. 2003; Stevanato et al. 2004; Bouranis et al. 2006).

Among root traits, root tips play a role as "plant command center" for the perception of the exogenous stress signals originating in the rhizosphere and for their conversion to endogenous phytohormone signals (Aiken and Smucker 1996; Forde 2002; Baluška et al. 2004). The synthesis of hormones in the root tips also seems to be regulated by translocation of other molecular signals, such as glucose, fructose and fructan (Gibson 2004). The translocation of soluble carbohydrates in the root tips has relevance not only for energy balance (ATP synthesis) but also for root

growth. In *Arabidopsis thaliana*, the concentration of glucose and fructose in the apical region of the primary root appears to influence the entire root architecture (Freixes et al. 2002), leading to adjustments (plasticity) of the root apparatus at morphological and physiological levels, which allow the plant to adapt to changing soil conditions (Hermans et al. 2006; Osmont et al. 2007). The evaluation of the root plasticity patterns or "reaction norms" (class of phenotypes produced by a genotype in different environments) represents one of the most promising approaches to identify adaptive traits to edaphic stress (Bell and Sultan 1999; Lynch and St. Clair 2004; Wu et al. 2004; Sorgonà et al. 2007). Wild and ruderal plants often display high levels of phenotypic plasticity for adaptation to edaphic heterogeneity of their habitat (Leiss and Müller-Schärer 2001; Pigliucci 2001). Edaphic variations may drive different patterns of selection within and between natural populations with a differentiation of specific genotypes (Slatkin 1973; Baythavong 2007).

Associating molecular markers with root phenotypes under stress and non-stress is a promising means to assess genetic diversity and relationships among genetic pools (McGrath et al. 2007). Among molecular markers, amplified fragment length polymorphism (AFLP) is one of the most widely applied DNA fingerprinting technique being robust and reproducible (Vuylsteke et al. 2007). Here we compared patterns of genetic diversity deduced from molecular markers and root morpho-physiological traits among a sea beet and a ruderal beet population collected along the Adriatic coast and a sugar beet variety. We have identified root morpho-physiological traits that are influenced by low sulfate availability.

Materials and methods

Plant material

Three populations of beets were examined: a sea beet population (*B. v.* ssp. *maritima*), a ruderal beet population and a sugar beet cultivar (*B. v.* ssp. *vulgaris*). The two sea and ruderal wild beet populations were collected in two different sites (20 km apart) of Po Delta Natural Park (Italy). Seed samples were collected from 20 randomly selected individual plants from each population at intervals >2 m between individuals at the

end of July in 2 years (2003–2004). The sugar beet cultivar was a commercial variety (Shannon) registered by Lion Seeds Ltd. Five individual plants were used for genotyping analysis. The sample size was chosen based on a previous study on cultivated and wild beet accessions (Bartsch et al. 2002).

The morpho-physiological analysis was performed on 60 seedlings of each accession per five replicates.

Genotyping analysis

DNA was extracted following the CTAB method described by Hoisington (1992) from young leaves of 2-month-old seedlings. AFLP analysis was performed at Keygene N. V. following the protocol of Vos et al. (1995) using EcoRI and MseI restriction enzymes (New England BioLabs, Beverly, MA). Selective amplification was carried out using eight different primer combinations (EcoRI-ACA/MseI-ACA; EcoRI-ACA/MseI-ACG; EcoRI-ACA/MseI-AGA; EcoRI-ACA/MseI-CAT; EcoRI-ACA/MseI-CCA; EcoRI-ACG/MseI-ACA; EcoRI-AGC/MseI-ACT; EcoRI-AGC/MseI-CAG). Radio-labeled fragments were separated on 6% poly-acrylamide gels and scored as categorical data (presence and absence).

Morpho-physiological analysis

Seeds were surface-sterilized by immersion for 10 min in 1% (v/v) sodium hypochlorite, rinsed several times with distilled water, then imbibed in aerated, deionized water at 22°C for 12 h. Seeds were transferred to two layers of filter paper moistened with distilled water in petri dishes placed in a germinator at 25°C in the dark for 48 h.

Three-day-old seedlings with 10 ± 2 mm long seminal roots were transplanted in plastic tanks over an aerated solution containing 200 μM $Ca(NO_3)_2$, 200 μM KNO_3, 200 μM $MgSO_4$, 40 μM $KH2PO_4$ and microelements (Arnon and Hoagland 1940). The nutrient solution was replaced daily. The tanks were placed in a growth chamber at 25/18°C and 70/90% relative humidity with a 14 h light (60 W m^{-2}) and 10 h dark cycle. On the 6th day, seedlings were transferred to sulfate-depleted hydroponic solutions where $MgSO_4$ was replaced with $MgCl_2$. Control treatments were not sulfate deprived.

Primary root length of individual seedlings was manually measured each day after initiation of nutritional stress until seedlings were 12-day old. The primary root elongation rate was calculated for each day from the difference in root length between two different measurements on successive days.

Root morphological traits were evaluated on 12-day-old seedlings by means of a scanner-based image analysis system (WINRHIZO Pro, Regent Instruments, QC, Canada) that controls scanning, digitizing and analysis of root samples. Before measurements, the entire root systems of individual plants were stained for 15 min with 0.1% (w/w) of toluidine blue (Sigma–Aldrich, Montréal, QC) to increase contrast, and washed free of stain with deionized water. The stained root systems were floated in 3 mm of water in a 0.3×0.2 m Plexiglas tray, and lateral roots were spread so as to minimize root overlap. The tray was placed on the glass surface of a STD-1600 EPSON scanner set to a scanning resolution of 1,200 dpi. Images were acquired and used to determine the total root length (cm), the number of root tips, and the average root diameter (in three ranges: <0.5 mm, between 0.5 and 1 mm and >1 mm).

Glucose and fructose concentrations were determined from apical regions of primary roots (0.5 mm long) harvested at the beginning of the photoperiod 6 days after initiation of sulfate deprivation. Each sample was rapidly rinsed in water then placed in 200 μl 80% ethanol at 80°C for 15 min. Extraction was repeated and the two extracts were pooled, and then dried under vacuum (SC110A; Savant Instruments Inc., Farmingdale, NY, USA). Extracts were re-suspended in 150 μl of distilled water and soluble carbohydrates (glucose and fructose) were quantified using a Technicon Instruments AutoAnalyzer (Pulse Instrumentation, 1992, Canada) following manufacture's procedures. Glucose and fructose concentration were quantified using calibration curves.

Data analysis

Morpho-physiological data were subjected to ANOVA using PLABSTAT software (Utz 1995). The effects of accession, treatment and accession-by-treatment interaction were evaluated. Least significant difference test (LSD) at the 0.01 probability level was used to compare data from different factors. Phenotypic plasticity was determined for each root trait by measuring the percentage variation of the trait observed after sulfate deprivation (−S) on the steady condition (+S),

according to the formula adapted from Zhu et al. (2005):

$$100 \times \left(\frac{\text{root measure under nutritional deprivation} - \text{root measure under steady condition}}{\text{root measure under steady condition}} \right).$$
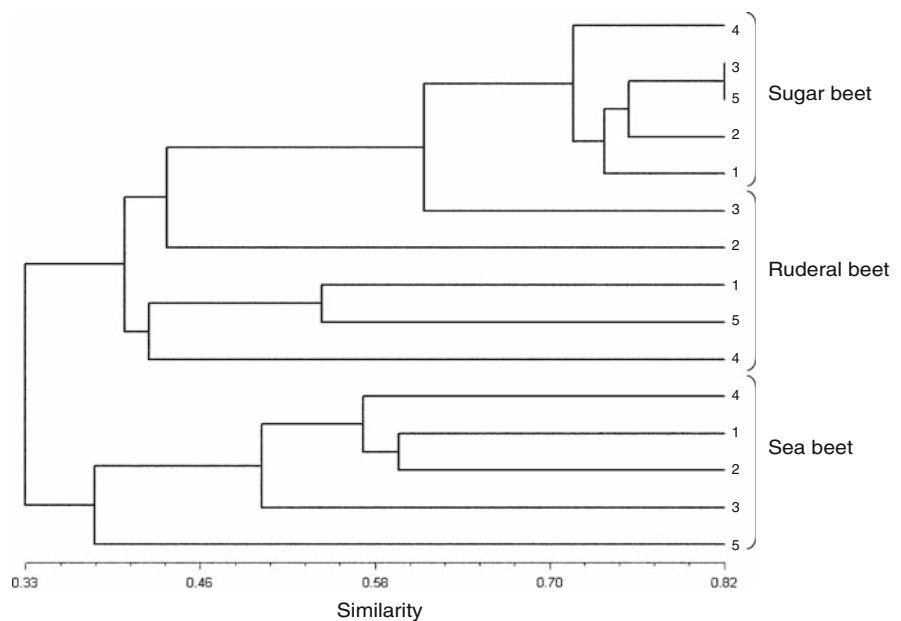
Morpho-physiological and genotypic data were used to carry out similarity analyzes among the three genotypes using Numerical Taxonomy System software, Version 2.1 (NTSYSpc, Exeter Software, Setauket, New York, USA) (Rohlf 2000). Similarity matrixes for AFLP data were calculated using Jaccard (J) coefficients (Jaccard 1908) where those for phenotypic data were calculated using Euclidian distances. To visualize the relationship between genotypes, the similarity matrix based on the J coefficient and Euclidian distances were used for the construction of dendrograms and principal co-ordinate analysis (PCO) using SAHN (Sequential Agglomerative Hierarichal Nested) and UPGMA (Unweighted Pair-Group Method, Arithmetic average) procedure (Sokal and Michener 1958). Cophenetic values were calculated from both AFLP- and phenotype-based dendrograms and were compared with their respective similarity matrices to evaluate whether the data in the similarity matrices were represented in the phenograms.

Mantel's Z-test (Mantel 1967) was used to compare AFLP and phenotypic dendrograms.

Results

Molecular analysis

Eight AFLP primer combinations generated a total of 194 polymorphic bands between individuals, of which 22 were specific to the sea beet accession, 17 to the ruderal beet accession and only 4 specific to the cultivated beet. The dendrogram based on the J coefficient clustered these accessions into three distinct groups (Fig. 1). Genetic similarities ranged from 38% between sea beet and other beets to 72% among sugar beet individuals, and greater heterozygosity was observed in sea beets relative to the other beets. Ruderal beets clustered more closely with sugar beet, consistent with appearance and its presumed ancestry involving both cultivated and wild beets. PCO plot analyzes showed a clear differentiation between the three beet accessions, with the first and second principle components explaining 19 and 14%

Fig. 1 Dendrogram of sugar beet, ruderal beet and sea beet accessions revealed by UPGMA cluster analysis based on Jaccard similarity coefficients obtained from AFLP markers

of the total phenotypic variation, respectively (Fig. 2).

Morpho-physiological analysis

Morpho-physiological analysis of root traits showed significant differences among the three accessions,
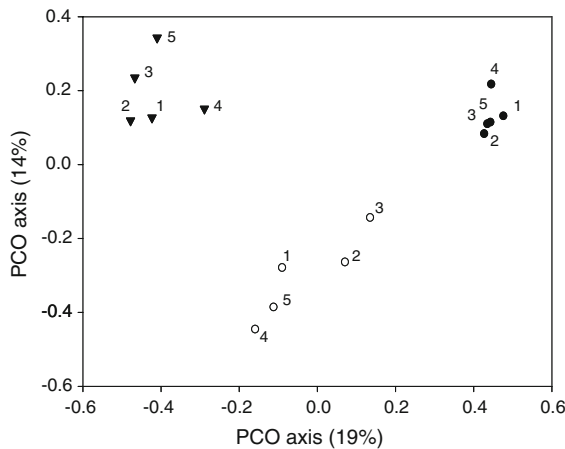

Fig. 2 Principal coordinate (*PCO*) analysis of sugar beet (●), ruderal beet (○) and sea beet (▼) accessions based on Jaccard similarity coefficient derived from AFLP markers

Fig. 3 Root traits of sugar beet (●), ruderal beet (○) and sea beet (▼) accessions grown in the complete solution (+S) and under sulfate deprivation (−S). Each data point is the mean of five replicates and 60 seedlings per replicate, with *error bars* representing the standard error of the mean. Means within accessions followed by a *different letter* are significantly different at the 0.01 probability level
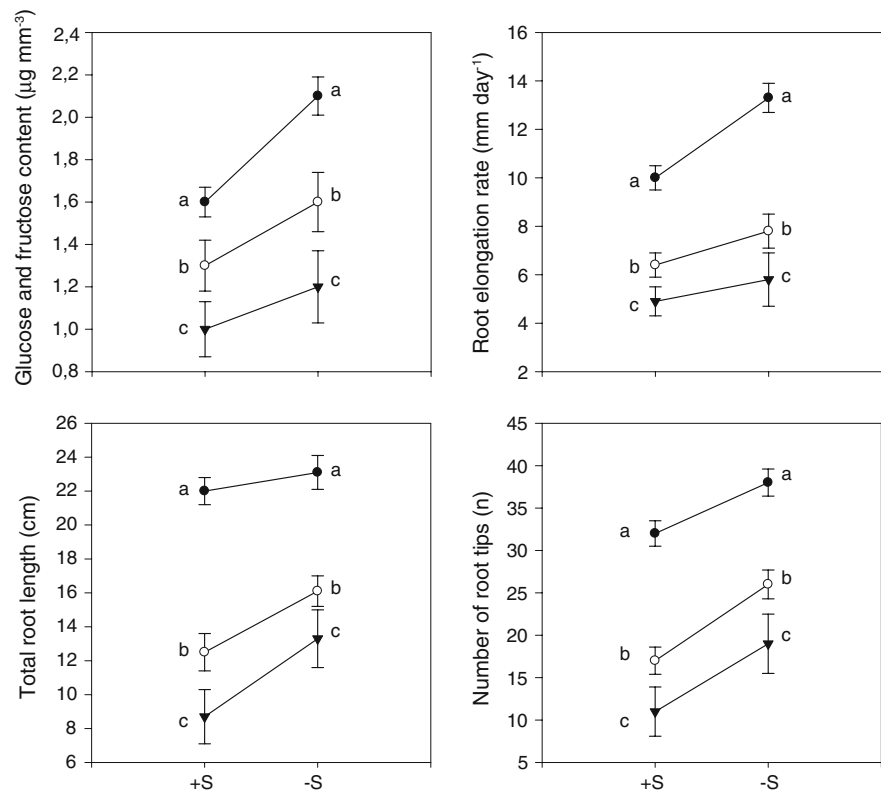
both in complete (+S) and sulfate-depleted (−S) conditions for all root traits examined (Fig. 3). ANOVA showed significant effects ($P < 0.01$) both of nutrient treatment (level of phenotypic plasticity) and of accession-by-treatment interactions (intraspecific genetic differences for plasticity) (Table 1). All measured traits increased under sulfate deprivation in each of the accessions.

The cultivated variety (sugar beet) displayed the highest values of glucose and fructose concentration in the root tips, primary root elongation rate, total root length, and number of root tips (Fig. 3), and the highest increase values for root tip glucose and fructose content and primary root elongation rate (Table 2). The wild accession (sea beet) showed the lowest values for all traits evaluated (Fig. 3) but the highest increases for total root length and number of root tips after S-starvation (Table 2). The ruderal beet accession had intermediary values among the sea and sugar beet accessions. Morphometric differences among 12-day-old seedlings of the three accessions are reported in Fig. 4.

Splitting total root length among the three diameter classes (fine: <0.5 mm; medium: between

Table 1 Results of ANOVA: effects of accession, treatment and two-factor interaction on the root traits evaluated ($P < 0.01$)

Variable	Accession (A)		Treatment (T)		$A \times T$	
	F-value	P-value	F-value	P-value	F-value	P-value
Glucose and fructose content	46.3	<0.01	62.9	<0.01	2.8	<0.01
Root elongation rate	35.9	<0.01	58.1	<0.01	3.9	<0.01
Total root length	37.2	<0.01	41.0	<0.01	2.4	<0.01
Number of root tips	53.4	<0.01	61.3	<0.01	2.8	<0.01
Length of fine roots	51.0	<0.01	59.4	<0.01	3.3	<0.01
Length of medium roots	49.2	<0.01	47.8	<0.01	1.2	NS
Length of large roots	43.5	<0.01	4.5	NS	0.6	NS

NS not significant

Table 2 Percentage variation of root traits measured after sulfate deprivation (−S) compared to values determined on steady nutrient supply

Accession	Glucose and fructose content (%)	Root elongation rate (%)	Total root length (%)	Number of root tips (%)
Sugar beet	+31 a	+33 a	+5 c	+19 c
Ruderal beet	+23 b	+22 b	+28 b	+53 b
Sea beet	+20 b	+18 b	+52 a	+73 a

Values followed by different letter are significantly different ($P < 0.01$)

0.5 and 1 mm; large roots >1 mm), significant differences were observed among the three accessions with sugar beet the highest and sea beet the lowest when grown under complete nutrient solution (Fig. 5). Sugar beet showed significant higher values for all root diameter classes whereas significant differences were found only for large roots between ruderal and sea beet after sulfate starvation (Fig. 5).

An increase of total length of fine roots and a decrease of the length of medium roots were observed in the three accessions, whereas large roots did not show significant variation (Table 3). The sea beet accession displayed a significantly higher increase (+115%) of the fine root length in response to S-starvation with respect to ruderal beet and sugar beet which, respectively, showed an intermediate (+78%) and lower increase (+32%). No significant differences were observed for medium and large root length among the three accessions (Table 3).

Cluster analysis of the four morpho-physiological root traits revealed three distinct clearly separated groups (Fig. 6). The Euclidean distance coefficients ranged from 0.11 (cultivated beet) to 0.48 (sea beet). Sea beet displayed a higher genetic diversity for root phenotypic traits with respect the other accessions. PCO plot from the Euclidean distance matrix indicated that beet accessions are clearly differentiated and the first and second components explained 21 and
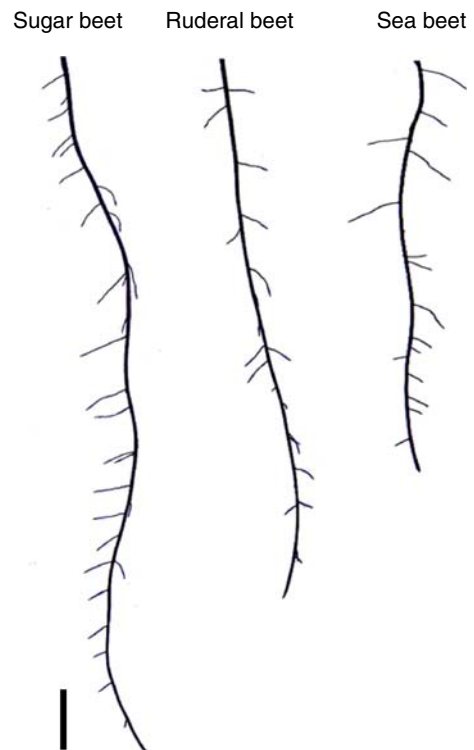
Fig. 4 Scans of roots of 12-day-old seedlings grown in hydroponics. *Bar* = 1 cm

17% of the total phenotypic variation, respectively (Fig. 7). Comparison of dendrograms (Figs. 1, 6) based on molecular and morpho-physiological data
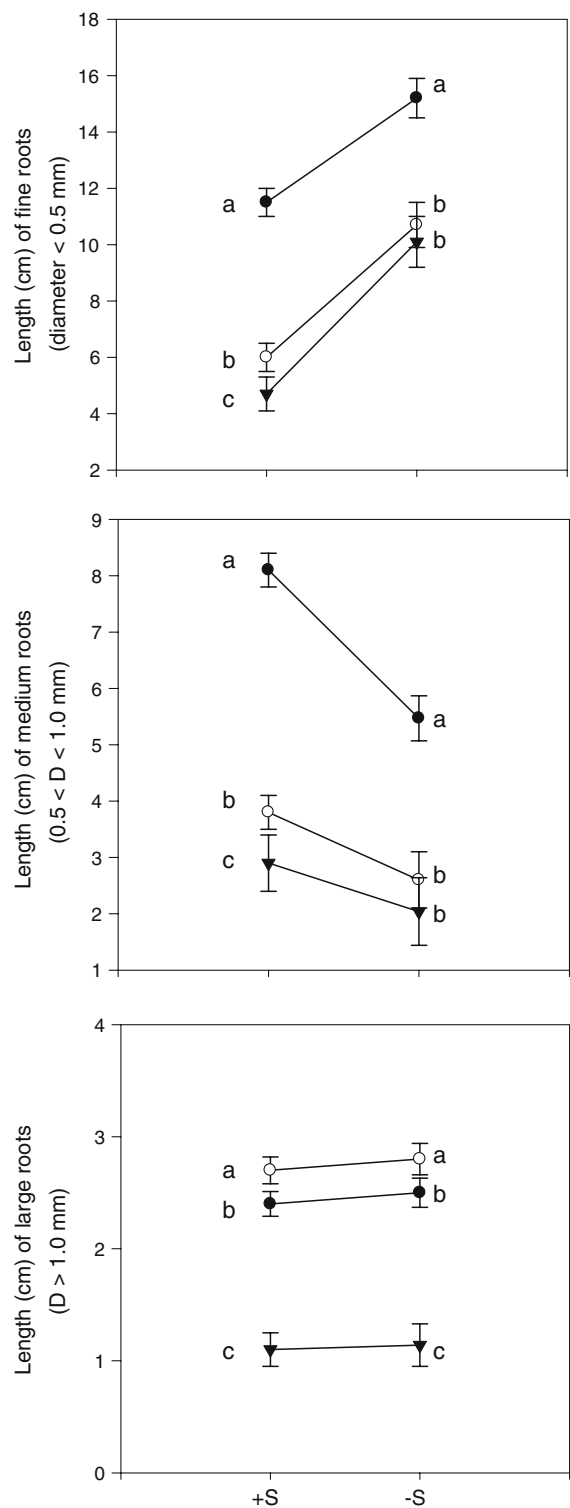
Fig. 5 Root length distribution among diameter ranges (*D*) of ▶ sugar beet (●), ruderal beet (○) and sea beet (▼) accessions grown in the complete solution (+S) and under sulfate deprivation (−S). Each data point is the mean of five replicates and 60 seedlings per replicate, with *error bars* representing the standard error of the mean. Means within accessions followed by a *different letter* are significantly different at the 0.01 probability level

using Mantel's *Z*-test indicated a significant correlation between them (*P* < 0.01).

Discussion

Amplified fragment length polymorphism analysis clearly distinguished between the three beet accessions and confirmed its usefulness for phylogenetic and fingerprinting studies (Joy et al. 2007; Vuylsteke et al. 2007). The intermediate position of ruderal beets between the cultivated and sea beets is in accord to the results obtained by Desplanque et al. (1999) and Fénart et al. (2008) on beet accessions of various French geographic origins. Bartsch et al. (2002) showed that the average genetic diversity within sugar beet cultivars was much lower than that found for Po Delta sea beets, and the cultivated beet showed a narrower genetic base relative to undomesticated beets. A higher level of wild beet polymorphism, with respect to domesticated beet by means of microsatellite (SSRs) markers, was highlighted by Richards et al. (2004). Therefore, our results confirm that breeding practices have impoverished the sugar beet genetic pool as indicated by McGrath et al. (1999) with the probable loss of alleles influencing response to environmental stresses (Frese et al. 2001).

The diversity patterns shown by molecular analysis appeared to be influenced not only by selection and breeding practices for sugar beet but also by the adaptation to the highly variable selection pressures that occur in the sea and ruderal beet habitats. Lososová et al. (2006) suggested that agricultural habitats are environmentally more homogenous than ruderal/natural habitats due to traditional agronomic practices. Hamrick and Allard (1972) and Hamrick and Holden (1979) showed that allele polymorphism is more easily maintained in a heterogeneous environments, and Hedrick (2006) reported in several plant and animal species that polymorphic allele frequencies increased with environmental heterogeneity.


Morpho-physiological data indicated that sea, ruderal and cultivated beet differed greatly in root development, confirming results of Gallardo et al.

Table 3 Percentage variation of the distribution of total root length among diameter ranges (D) examined after sulfate deprivation ($-S$) compared to values observed on steady nutrient supply

Accession	Total root length (cm)		
	$0.0 < D < 0.5$ mm (%)	$0.5 < D < 1.0$ mm (%)	$1.0 < D < 1.5$ mm (%)
Sugar beet	+32 c	−48 NS	+3 NS
Ruderal beet	+78 b	−46 NS	+3 NS
Sea beet	+115 a	−42 NS	+4 NS

Values followed by different letter are significantly different ($P < 0.05$)

Fig. 6 Dendrogram of sugar beet, ruderal beet and sea beet accessions revealed by UPGMA cluster analysis based on Euclidean distance derived from morpho-physiological data
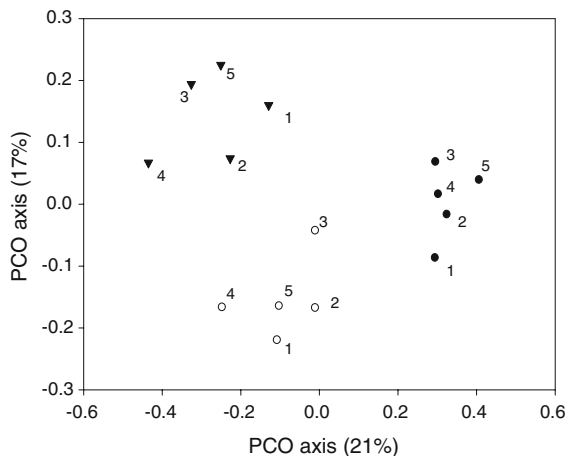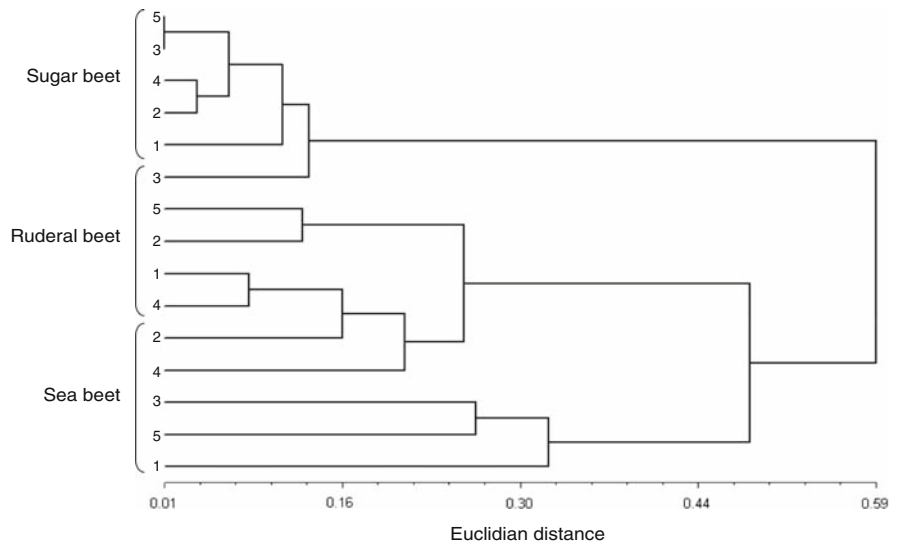
Fig. 7 Principal coordinate (*PCO*) analysis of sugar beet (●), ruderal beet (○) and sea beet (▼) accessions based on Euclidean distance derived from morpho-physiological data

(1996) who showed significant effects of domestication on seedling root morphology and architecture between cultivated lettuce (*Lactuca sativa* L.) and its wild progenitor (*Lactuca serriola* L.). Bouranis et al. (2006) showed that S-deprived maize plants increased root proliferation, the sea and cultivated beet accessions showed analogous direction of plasticity of root morpho-physiological traits after sulfate deprivation but they displayed contrasting root adaptive strategies to S-starvation, while the ruderal beet accession appeared to be intermediate.

The sugar beet root adaptive strategy to S-deprivation appears largely based on the increase of reducing sugars in the root tips and thus favoring root elongation rate (Freixes et al. 2002; Choi et al. 2007). Such traits might be advantageous in exploiting "deep soil resources". For instance, the taproot elongation of wild lettuce in response to water stress allowed water to be extracted from greater depth than cultivated lettuce (Gallardo et al. 1996). In addition, the maize genotype characterized by the highest seminal root elongation rate after S-starvation, also showed the highest root length density in deep soil layers (Vamerali et al. 2003). Similarly, Manschadi

et al. (2006) observed that a wheat (*Triticum aestivum* L.) genotype emphasizing the vertical growth of its seminal roots displayed a deeper root apparatus and greater soil water extraction than a standard wheat cultivar.

The adaptive strategy to S-deprivation of sea beet, which displayed the lowest increase in the rate of primary root elongation and the greatest increase in the number of root tips, the total root length and the length of fine roots (which appeared the most plastic character) appears to be a reflection of horizontal root growth (e.g. more root branching and growth of these branches). These traits might allow a competitive advantage for soil resource acquisition in the topsoil. Eshel and Waisel (1996) and Reidenbach and Horst (1997) pointed out that the root tips are key traits in plant competitiveness for nutrients and are the most active part of the root apparatus in acquiring water and nutrients. Furthermore, high total root length is relevant for nutrient acquisition at low nutrient availability (Caldwell et al. 1991; Ryser and Lambers 1995). An increase in fine roots is an important mechanism of plant adaptation to nutritional stress because fine roots are highly sensitive to nutrient fluctuations (Wu et al. 2004), can be rapidly deployed into nutrient-rich patches (Wijesinghe et al. 2001), and are very efficient in exploring the soil per unit of metabolic investment in root biomass (Eissenstat 1992). An increase of 22% in root fineness resulted in a threefold increase in phosphorous uptake in phosphorus-deficient rice plants (Wissuwa 2003). This "root horizontal growth strategy", when adopted by bean and maize genotypes in low phosphorus soils, allowed a superior phosphorus acquisition (Liao et al. 2001; Zhu et al. 2005).

The significant correlation between molecular and morphophysiological dendrograms in the three beet accessions was also found in *Solanum melongena* and *Sesamum indicum* L., respectively by Furini and Wunder (2004) and Ali et al. (2007). However, several studies have shown that molecular differences are not always concordant with phenotypic traits (Rana et al. 2005; Sorkheh et al. 2007; Sreekumar et al. 2007).

In conclusion, these root strategies are of great ecological and functional significance allowing plants to adapt to heterogeneous distribution of soil resources. Undomesticated beets of the Po Delta appear to be a source of genetic resistances to adverse environmental stresses (Stevanato et al. 2001; Biancardi et al. 2002). Selection for a sugar beet root ideotype aimed at improving the nutrient-capturing capacity should consider the combination of cultivated and sea beet adaptive strategies. An enhanced plasticity for key root traits such as root elongation rate, number of root tips, and fine root length might improve sugar beet adaptive ability to different patterns of nutrient supply and crop productivity. Further morpho-physiological and molecular studies are required to introgress these traits in sugar beet cultivars for low-sustainable farming.

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